In re Application of: Michal AMIT et al.
Serial No.: 10/537,784
Filed: 06/06/2005
Office Action Mailing Date: 01/28/2008

Examiner: Deborah Crouch
Group Art Unit: 1632
Attorney Docket: 29606

REMARKS

Reconsideration of the above-identified application in view of the amendments above and the remarks following is respectfully requested.

Claims 153-229 are in this Application. Claims 218-220 have been withdrawn from consideration. Claims 164, 165, 168, 169, 182, 183, 194, 195, 198, 199, 210, 211, 214, 215, 226 and 228 have been rejected under 35 U.S.C. § 112. Claims 153, 155, 156, 159-163, 166, 170-177, 180, 181, 184, 188, 189, 200-203, 209, 212, 216, 217, 221-223, 225 and 227 have been rejected under 35 U.S.C. § 102. Claims 153-156, 159-163, 166-167, 170-181, 184-203, 206-209, 212-213, 216-217, 221-223, 225 and 227 have been rejected under 35 U.S.C. § 103. Claim 180 has been cancelled herewith. Withdrawn claim 219 has been amended herewith. Claims 153, 155, 158, 161, 168-171, 176, 181-185, 188-190, 193-197, 203, 206, 209-213, 221, 225-227 and 229 have been amended herewith. New claims 230-237 have been added herewith.

Amendments To The Claims

35 U.S.C. § 112 Rejections

The Examiner has rejected claims 164, 165, 168, 169, 182, 183, 194, 195, 198, 199, 210, 211, 214, 215, 226 and 228 under 35 U.S.C. 112, first paragraph, as failing to comply with the enablement requirement. The Examiner states that the claims contain subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

Specifically, the Examiner states that the claims are drawn to methods of culturing ES cells or human ES cells, and ES or human ES cell cultures where the culture media contains TGFβ1 or LIF, however, the art (Bodnar, Schuldiner, Thompson) taught that neither of these growth factors can maintain ES cells in a proliferative, pluripotent and undifferentiated state; and that the specification

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discloses that hES cells have began to differentiate at six days in culture when the media contains TGFβ1; βFGF; LIF and TGFβ1; or LIF and βFGF (Page 39, lines 20-23), thus TGFβ1 or LIF are not sufficient to maintain primate ES cells in a proliferative, pluripotent and undifferentiated state as claimed. The Examiner's rejections are respectfully traversed.

In order to expedite prosecution of this case, Applicants have elected to amend claims 153, 155 and 219 to recite a culture medium which comprises *"TGFβ1 and bFGF"* (Emphasis added); amend claims 168, 181, 193, 209, 225, and add new claim 235 to include LIF in a dependent manner, for which sufficient descriptive support is provided in the instant application, see e.g., the TF and TLF culture media described in Pages 28 (line 1) – 29 (lines 1-4) and Page 39 (lines 17-20 and 24-27), to thereby overcome Examiner's rejections.

Similarly, claims 170, 171, 189, 203, 219 and 221 have been amended to include the limitation of "*"TGFβ1 and bFGF"* (Emphasis added).

The Examiner has further rejected claim 229 under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for a method of maintaining hES cells in an undifferentiated, pluripotent and proliferative state under culture conditions devoid of feeder cells, the method comprising culturing hES cell on an *extracellular* matrix, in the present of media comprising 15% serum replacement, 0.12 ng/ml TGFβ1, 1000 u/ml LIF and 4 ng/βFGF, wherein said cells are maintained for at least 56 passages with a doubling time of at least 25 hours, does not reasonably provide enablement for a method of maintaining hES cells in an undifferentiated, pluripotent and proliferative states under culture conditions devoid of feeder cells, the method comprising culturing hES cell on a matrix, in the present of media comprising 15% serum replacement, 0.12 ng/ml TGFβ1, 1000 u/ml LIF and 4 ng/ml βFGF, wherein said cells are maintained for at least 56 passages with a doubling time of at least 25 hours. The Examiner states that the specification does not enable any person

skilled in the art to which it pertains, or with which it is most connected, to make and use the invention commensurate in scope with these claims. The Examiner's rejections are respectfully traversed.

In order to expedite prosecution of this case, Applicants have elected to amend claim 229 to recite "an extracellular matrix" instead of "matrix" according to Examiner's suggested language, to thereby overcome Examiner's rejections.

In view of the above claim amendments and remarks Applicants believe to have overcome the 35 U.S.C. § 112, first paragraph rejections.

35 U.S.C. § 102 Rejections

The Examiner has rejected claims 153, 155, 156, 159-163, 166, 170-177, 180, 181, 184, 188, 189, 200-203, 209, 212, 216, 217, 221-223, 225 and 227 under 35 U.S.C. 102(b) as being anticipated by WO 99/20741 (Bodnar). Specifically, the Examiner states that Bodnar teaches a method of establishing a xeno-free, feeder cell-free hESC cell line, a method of propagating a species ESC line in an undifferentiated, pluripotent and proliferative state and methods of maintaining hESC by the culture of PSC43 rhesus monkey ES cells in a media comprising 20% FCS on a MEF matrix or a fibronectin matrix in the presence of bFGF; that Bodnar teaches culturing PSC43 cells in media supplemented with bFGF or LIF; that Bodnar teaches the culture of PSC43 cells on a matrix in species-conditioned media; that Bodnar teaches a culture medium where the culture is substantially free of xeno and feeder cell contaminants; and that Bodnar teaches that the method taught can be used to maintain human ES cells. In addition, the Examiner states that the specification offers no definition of the term "xeno-free" and thus the lack of feeder is seen rendering the cell culture of Bodnar "xeno-free", free of cell contaminants. The Examiner further states that it is noted that certain claims, such as claim 170, use terminology "capable of" which does not require a positive attribute to the product or method claimed as the

only requirement is that the method or the product under certain conditions may have the limitation claimed. In addition, the Examiner states that since the method steps claimed and those of Bodnar are the same, it is an inherent property of Bodnar's cells that the cell would comprise at least 85% undifferentiated species ES cells and have a doubling time of at least 25 hours. The Examiner's rejections are respectfully traversed.

With respect to Examiner's assertion that Bodnar et al. teach culturing ESCs in a medium which comprises bFGF or LIF, Applicants point out that in sharp contrast to Bodnar et al., the instant application as now claimed pertains to the use of a culture medium which comprises TGFβ1 and bFGF, which is not taught or suggested by Bodnar et al. Therefore it is Applicants position that Bodnar et al could not anticipate the claimed invention.

With respect to Examiner's assertion that since the specification does not offer a definition for "xeno-free" the lack of feeder cells in Bodnar et al. renders the cell culture of Bodnar "xeno-free", Applicants point out that the phrase "xeno-free" should be interpreted according to the interpretation that would be given by one possessing the ordinary level of skill in the pertinent art at the time the invention was made (see MPEP 2173.02). Since the phrase *"xeno-free"* is art-accepted (e.g., about 4,190 results are obtained when searching the web using the Google™ search tool at http://www.google.com) it is Applicants' position that no definition for the phrase is required. Thus, as "xeno" is a prefix based on the Greek word "xenos" (which means a foreigner; see http://www.searchgodsword.org/lex/grk/view.cgi?number=3581), it is clear that when used with respect to a certain species (e.g., homo sapiens) conditions which are *xeno-free* are those devoid of other *i.e.*, foreigner species (e.g., non-human, animal) contaminants (see e.g., Page 36, lines 14-16 in the instant application as filed; and Richards M., et al., 2004, attached herewith). Since Bodnar et al. culture PSC43 cells (Rhesus monkey embryonic stem cells) on a mouse-derived feeder-free matrix (made from mouse embryonic fibroblast; See Page 20, lines 13-18 in Bodnar et al.) in

the presence of a medium supplemented with either a mouse embryonic fibroblast conditioned medium (See Page 19, lines 4-16 in Bodnar et al.) or fetal-bovine serum (see Page 20, from line 27, through Page 21, line 6; Page 23, from line 28, through Page 24, line 6; and Page 19, lines 6-11 in Bodnar et al.) it is Applicants position that Bodnar et al. do not teach conditions devoid of xeno contaminants since they used medium derived from one species (e.g., mouse or bovine) to culture cells of another species (monkey).

Notwithstanding the above, and in order to render explicit what was already implicit, Applicants have elected to amend claims 170, 171 and 203 to include the limitation of "devoid of xeno contaminants". Support for such amendments can be found for example in Pages 14 (lines 10-17), 8 (lines 22-23), 15 (lines 9-13), 18 (lines 18-20), 19 (lines 13-25), 21 (lines 5-8) and 22 (lines 20-25) of the instant application as filed.

With respect to Examiner's assertion that the claimed method steps are the same as those of Bodnar and therefore it is an inherit property of Bodnar's cells to comprise at least 85 % of undifferentiated cells and have a doubling time of at least 25 hours, Applicants point out that since, as stated above, Bodnar did not teach the use of a culture medium which comprises TGFβ1 and bFGF, the claimed method steps are entirely different from those of Bodnar and therefore it is NOT an inherit property of the cells of Bodnar to comprise at least 85 % undifferentiated cells and have a doubling time of 25 hours.

Altogether, it is Applicants position, that the art of Bodnar et al. could not anticipate the subject matter of the invention as now claimed.

In view of the above claim amendments and remarks Applicants believe to have overcome the 35 U.S.C. § 102 rejections.

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35 U.S.C. § 103 Rejections

The Examiner has rejected claims 153, 154, 167, 170, 176, 178-181, 185-187, 190-193, 196, 197, 203, 206-209 and 213 under 35 U.S.C. 103(a) as being unpatentable over WO 99/20741 (Bodnar) in view of Amit et al. (2000, Dev. Biol. 227: 271-278). Specifically, with respect to Amit et al., the Examiner states that Amit et al. teach culturing hES cells on MEF feeder cells in a media containing 20 % serum replacement and 4 ng/ml bFGF and cloning of H9 cells. Thus, the Examiner states that at the time of the instant invention it would have been obvious to the ordinary artisan to establish a xeno-free, feeder cell-free ES or hES cell line, propagate a species ES cell line in an undifferentiated, pluripotent and prolifetative state and a cell culture comprising undifferentiated, pluripotent proliferative hES cells by modifying the primate ES cell line culture as taught by Bodnar and also taught by Bodnar to be useful for culturing human ES cell lines, with the modifications of serum replacement, culturing in the presence of 4 ng/ml bFGF and cloning the ES cells, each taught by Amit and for the motivation provided by Amit. Examiner's rejections are respectfully traversed.

Applicants point out that a *prima facie* case of obviousness is not properly set, since Bodnar and/or Amit do not teach culturing embryonic stem cells under feeder cell-free conditions in the presence of a culture medium which comprises TGFβ1 and bFGF as claimed, thereby failing to provide all the claimed features.

Thus, as stated above with respect to the 102 rejections, Bodnar et al. did not teach or suggest culturing the ESCs with TGFβ1 and bFGF as claimed. Similarly, with respect to Amit et al., Applicants point out that Amit et al. cultured the hESCs on mouse feeder cells in the presence of a medium supplemented with bFGF, but did not teach or suggest culturing the cells on feeder-free conditions in a medium supplemented with TGFβ1 and bFGF. Thus, it is Applicants position that the instant application as now claimed invention is novel and non-obvious over the art of Bodnar et al. and/or Amit et al.

The Examiner has further rejected claims 153, 156-158, 203-205, 221, 223 and 224 under 35 U.S.C. 103(a) as being unpatentable over Bodnar in view of Sigma Catalog, 1992, Page 1389, Cat. Nos. F2518 and F2006. With respect to the Sigma Catalogue the Examiner states that Sigma teaches human fibronectin isolated from human foreskin and human plasma, and their uses at cell attachment matrices in cell culture. The Examiner further states that at the time of filing, it would have been obvious to the ordinary artisan to use human fibronectin in view of Bodnar teaching increases in hES cell proliferation when grown on fibronectin and the Sigma catalog teaching the availability of human plasma and foreskin fibronectin, and the use of fibronectin as a cell culture matrix, and that the substitution of one fibronectin for another to enhance growth, viability, pluripotency of hES cells is obvious absence results to the contrary. The Examiner's rejections are respectfully traversed.

Applicants point out that a *prima facie* case of obviousness is not properly set, since Bodnar and/or the Sigma catalogue do not teach culturing embryonic stem cells under feeder-free conditions which include a culture medium which comprises TGFβ1 and bFGF as claimed, thereby failing to provide all the claimed features.

Thus, as stated above with respect to the 102 rejections, Bodnar et al. did not teach or suggest culturing the ESCs with TGFβ1 and bFGF as claimed. With respect to the Sigma Catalogue, Applicants point out that the cited reference does not teach or suggest culturing ESCs in a medium which comprises TGFβ1 and bFGF as claimed.

Thus, it is Applicants position that the instant application as now claimed invention is novel and non-obvious over the art of Bodnar et al. and/or the cited Sigma catalogue.

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Support for claim amendments and new claims

Applicants have elected to amend without prejudice claim 153 to include the limitations of "obtaining stem cells of a human embryo". Support for the amendments can be found on Pages 16 (lines 32-33), 17 (lines 1-16) in the instant application as filed. Claims 170 and 188 have been amended in a similar manner.

In view of the above amendments and remarks it is respectfully submitted that claims 153-179, 181-217 and 221-237 are now in condition for allowance. A prompt notice of allowance is respectfully and earnestly solicited.

Respectfully submitted,

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Date: May 28, 2008

Enclosures:

- Petition for Extension (one Month)
- Additional Claims Transmittal Sheet
- Reference: Richards M., et al., 2004; Stem Cells, 22:779-789

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An Efficient and Safe Xeno-Free Cryopreservation Method for the Storage of Human Embryonic Stem Cells

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Original Article

An Efficient and Safe Xeno-Free Cryopreservation Method for the Storage of Human Embryonic Stem Cells

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Key Words. Closed straws · Cryovials · Human embryonic stem cells · Liquid nitrogen vapor Slow machine freezing · Undifferentiated · Vitrification

ABSTRACT

Human embryonic stem cells (hESCs) promise to revolutionize reparative medicine through their potential in developing cell replacement therapies for diseases like diabetes and parkinsonism. Most of the existing hESC lines available for research, including all National Institutes of Health–registered lines, have been derived and maintained on mouse embryonic fibroblast feeders in the presence of xenoproteins. For future clinical application, many more hESC lines derived and grown in current good manufacturing practice, good tissue culture practice, and xeno-free conditions need to be developed. Concurrently, effective cryopreservation methods that prevent or limit the accidental contact of hESCs with nonsterile liquid nitrogen during periods of long-term storage have to be formulated. We describe a safe, xeno-free cryopreservation protocol for hESCs involving vitrification in closed sealed straws using human serum albumin as opposed to fetal calf serum as the main protein source in the cryoprotectant and long-term storage in the vapor phase of liquid nitrogen. After thaw, hESCs exhibited high thaw-survival rates and low differentiation rates, remained pluripotent, and maintained normal diploid karyotypes throughout extended passage. The cryopreservation technique we describe here should complement xeno-free culture conditions for hESCs already in refinement and will prove very useful for the setting up of hESC banks throughout the world. *Stem Cells 2004;22:779–789*

INTRODUCTION

All current 78 National Institutes of Health–listed human embryonic stem cell (hESC) lines approved for U.S. government federal research funding have been derived and propagated on mouse embryonic fibroblasts (MEFs) and in the presence of culture medium containing animal-based ingredients. The use of a feeder layer of animal origin and animal components in the culture media substantially elevates the risk of the cross-transfer of viruses and other pathogens to the embryonic stem (ES) cells. Hence, safer current good manufacturing practice (CGMP) and good tissue culture practice (GTCP)-compliant hESC lines and differentiated hESC progenitors need to be derived for clinical application.

Several attempts at improving hESC culture conditions have been reported. These advances include the use of conditioned media together with Matrigel™ as an attachment substrate for hESC culture [1] and the derivation and propagation of hESC lines on human feeder layers [2–6]. These improvements are important steps forward in developing a CGMP-compliant protocol for the establishment of xeno-

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free clinically compliant hESC lines. The derivation of xeno-free CGMP-compliant hES lines also necessitates the development of a cryopreservation protocol that is effective and minimizes or restricts the possibility of cell line contamination in long-term liquid nitrogen (LN_2) storage.

Numerous reports have been published describing the contamination of frozen blood and cells with adventitious agents, primarily viruses, in LN_2 storage tanks [7]. The cross-contamination of frozen cell stock from one cell line with other cell types in LN_2 storage tanks has also been well documented [8]. Several viruses, including the hepatitis B virus, have also been reported to survive well in LN_2 [9, 10].

Two freezing protocols are currently used for hESCs. These include (a) the conventional slow stepwise programmed freezing method using cryovials (CVs) and storage in LN_2 and (b) a snap-freezing vitrification method using an open pulled straw (OPS) and storage in LN_2 [11].

Although controlled rate freezing is popular for most somatic cell types, its use for hESCs has been shown to result in low thaw-survival rates and low plating efficiencies [11], presumably because of ice crystal formation during the cooling process that will disrupt cell-cell adhesion. Also, CVs are traditionally used in controlled-rate freezing methods, and LN_2 seepage into such tightly sealed screw-cap CVs often occurs in long-term storage. Vitrification, on the other hand, which works on the principle of glass induction instead of ice crystal formation, is simple, quick, and inexpensive. It has also been proven to consistently yield higher plating efficiencies than controlled-rate freezing methods. However, the OPS vitrification method, although simple and efficient, is not an ideal protocol for cryopreserving CGMP-compliant hESC lines, because it is nonsterile [11]. This method involves direct contact of hESCs with LN_2 via the open end of the pulled straw, thereby increasing the possibility of hESC contamination and infection. Sterile sources of LN_2 are available, but maintaining aseptic conditions while working with LN_2 is costly, cumbersome, and impractical.

Current cryopreservation methods also use fetal calf serum (FCS) containing xenoproteins in the formulation of freezing/vitrification and thawing/warming solutions. Therefore, the development of an effective, safe, and sterile cryopreservation protocol is a prerequisite for the long-term storage of CGMP- and GTCP-compliant xeno-free hESC lines in stem cell banks.

Hence, in this report, we sought to refine an established vitrification strategy for hESCs and compared its efficiency with the conventional controlled-rate freezing method. We document the successful vitrification of hESCs in sealed closed straws (CSs), their storage in the vapor phase of LN_2 (VLN_2), and the substitution of FCS with human serum albumin (HSA) as the major protein source in the cryoprotectant.

Materials and Methods

A total of eight different freezing protocols (controls: OPS-FCS–liquid phase of LN_2 [LLN_2]; experimental: OPS-FCS-VLN_2, CS-FCS-LLN_2, CS-FCS-VLN_2, CS-HSA-LLN_2, CS-HSA-VLN_2, CV-FCS-LLN_2, CV-FCS-VLN_2) were compared at the same time for two proprietary hESC lines (HES-3 and HES-4) from ESI Pte Ltd, Singapore. The total number of colonies analyzed for all of the eight arms of the experiment was 372 and 361 for HES-3 and HES-4, respectively. To additionally confirm the efficacy of the CS-HSA-VLN_2 approach, we repeated the experiment for this arm on another hESC line, HES-2, together with the appropriate

Table 1. Mean ± standard error of the mean percent post-thaw survival and differentiation of human embryonic stem cells (hESCs) after cryopreservation using different protocols

	Control	Experimental						
hESC colonies	OPS-FCS-LLN_2	CS-HSA-VLN_2	CS-HSA-LLN_2	CS-FCS-LLN_2	CS-FCS-VLN_2	OPS-FCS-VLN_2	CV-FCS-LLN_2	CV-FCS-VLN_2
Grade A	52.3 ± 5.5	58.8 ± 3.0	47.6 ± 6.2	51.9 ± 7.6	55.0 ± 6.5	49.8 ± 4.1	0.0 ± 0.0	0.0 ± 0.0
Grade B	27.6 ± 0.6	29.5 ± 2.0	32.6 ± 1.6	23.6 ± 2.1	26.8 ± 8.6	25.2 ± 5.6	10.0 ± 10.0	8.2 ± 1.9
A + B	79.9 ± 5.0[a]	88.3 ± 2.4[b]	80.1 ± 7.7[b]	75.5 ± 9.7[b]	81.7 ± 2.1[b]	75.0 ± 9.7[b]	10.0 ± 10.0[c]	8.2 ± 1.9[d]
Grade C	19.7 ± 4.5	12.2 ± 2.8	19.1 ± 6.9	23.9 ± 10.4	18.4 ± 2.2	22.7 ± 9.9	58.7 ± 11.3	27.5 ± 2.5
Grade D	0.5 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	0.7 ± 0.7	0.0 ± 0.0	2.4 ± 0.2	31.3 ± 21.3	64.4 ± 4.4
C + D	20.1 ± 5.0	12.2 ± 2.8	19.1 ± 6.0	24.6 ± 9.7	18.4 ± 2.2	25.1 ± 9.7	90.0 ± 10.0	91.9 ± 1.9

Grade A colony indicates colony with >80% undifferentiated; grade B colony, colony with 50%–80% undifferentiated; grade C colony, colony with <50% undifferentiated; grade D colony, unattached, dead, or lysed colony.

[a,c; a,d; b,c; b,d] $p < .001$.

Abbreviations: CS, closed straw; FCS, fetal calf serum; HSA, human serum albumin; LLN_2, liquid phase of liquid nitrogen; OPS, open pulled straw; VLN_2, vapor phase of liquid nitrogen.

OPS controls. Sixty-one HES-2 colonies were analyzed. At least two replicates for each arm of the experiment and also for each cell line were performed over passages 25 through 30. The data for the entire experiment in percentage values are summarized in Table 1.

hESC Culture

HES-2, HES-3, and HES-4 cell lines, which are of different ethnic backgrounds, were grown on mitomycin-C–inactivated D551/CCL-110 (American Type Culture Collection, Bethesda, MD) fetal skin fibroblast feeders with standard hESC culture media, comprised of 80% high-glucose Dulbecco's modified Eagle's medium (DMEM) (vol/vol), 20% hyclone-defined FCS (vol/vol) (HyClone, Logan, UT), 2 mmol/l L-glutamine, 50 IU/ml penicillin, 50 μg/ml streptomycin, 1 × nonessential amino acids, 1 × insulin-transferrin-selenium G supplement, and 0.1 mmol/l β-mercaptoethanol (Invitrogen, Carlsbad, CA). hESC colonies were passaged by mechanically cutting small clumps of undifferentiated hESCs and transferring these fragments to fresh feeders at approximately 7- to 8-day intervals before the onset of hESC differentiation (for detailed protocols on hES culture, please refer to References 2 and 3).

Solutions for hESC Slow Freezing and Thawing and Vitrification and Warming

Slow Freezing in CVs

For slow freezing of hES colony fragments in CVs, conventional freezing medium comprising 90% FCS and 10% dimethylsulfoxide (DMSO; Hybrimax, sterile filtered, endotoxin, and hybridoma tested, Sigma D2650) was used. Alternatively, commercially available cell culture freezing media (Gibco, catalog # 11101-011) was found to be a suitable substitute.

Vitrification in Straws

The holding medium, ES-HEPES-HSA, consisted of 80% DMEM (vol/vol) (high-glucose DMEM, Invitrogen, catalog #11960-044) and 20% HSA (vol/vol) (HSA-solution™, Vitrolife, Goteberg, Sweden, #10064 containing purified HSA at 100 mg/ml), buffered to 20 mM HEPES (1 M HEPES solution, Invitrogen, catalog #15630-080). The holding medium with sucrose, ES-HEPES-HSA-sucrose, was comprised of 1 M sucrose solution in ES-HEPES-HSA medium (e.g., 3.42 g sucrose in 10 ml ES-HEPES-HSA). Vitrification solution 1 (VS1) was comprised of 80% ES-HEPES-HSA (vol/vol), 10% DMSO (vol/vol) (DMSO, hybrimax, sterile filtered, endotoxin and hybridoma tested, Sigma D2650), and 10% EG (vol/vol) (Ethylene Glycol, Sigma E9129). Vitrification solution 2 (VS2) was comprised of 30% ES-HEPES-HSA (vol/vol), 30% ES-HEPES-HSA-sucrose (vol/vol), 20% DMSO (vol/vol), and 20% EG (vol/vol). Warming solution 1 (WS1) was comprised of 80% ES-HEPES-HSA (vol/vol) and 20% ES-HEPES-HSA-sucrose (vol/vol). Warming solution 2 (WS2) was comprised of 90% ES-HEPES-HSA (vol/vol) and 10% ES-HEPES-HSA-sucrose (vol/vol).

All solutions were sterile filtered before use. HSA was substituted for FCS in experimental arms. FCS was retained as a protein source in controls. Sterile embryo straws, 250-mL volume (Pailette Cristal, 0459, #006433), were purchased from Cryo Bio System, Groupe I.M.V. Technologies, Paris. OPS straws were purchased from Demtek, Aarhus, Denmark. These commercial straws are sold sterile with one end sealed with a cotton plug.

Method for Slow Freezing and Thawing in CVs

Approximately 10 to 15 hES colony fragments were placed inside a 1-ml CV (Nunclon, Roskilde, Denmark) containing 500 μL of conventional freezing medium. The CV containing the hES colony fragments was then placed inside a programmable freezing machine (Planar, London, U.K.) equilibrated at a steady temperature of 4°C. The cooling cycle was set at a decrease of 1°C/min to –30°C. After a 5-minute holding period at –30°C, the CVs were plunged directly and stored in LN_2. CVs were thawed rapidly by removing a CV from LN_2 storage and plunging directly into a 37°C water bath.

Method for Vitrification and Thawing in OPSs

Protocols developed by Reubinoff et al. [11] for OPS vitrification and thawing were used.

Method for Vitrification and Warming in CSs

Vitrification

Dissected colony fragments of hESCs were vitrified in clumps of approximately 300–400 cells as soon as possible to prevent the clumps from sticking together. After dissection, hES clumps were washed once in modified phosphate-buffered saline (PBS+) (Invitrogen) and then transferred into an organ culture dish containing holding medium to wash off PBS.

Vitrification was performed at room temperature in a four-well sterile tissue culture dish (Nunclon) containing holding medium and vitrification solutions (wells one through three), as shown in Figure 1. Five to eight colony fragments were first transferred to well one of the four-well dish containing holding medium using a sterile glass Pasteur pipette (colony clumps can be kept in this solution for at least 20–30 minutes). An embryo straw was then loaded through the open end with a column of VS2 (~20 mm) using the load-

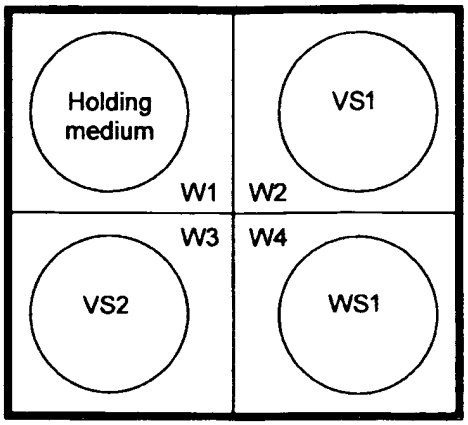

Figure 1. Line drawing of four-well dish showing vitrification solutions. Abbreviations: VS1, vitrification solution 1; VS2, vitrification solution 2; WS1, warm solution 1; W1, well 1; W2, well 2; W3, well 3; W4, well 4.

ing/unloading syringe device (Fig. 2). A column of air (~5 mm) was then aspirated. The straw attached to the syringe device was then left on a pipette rest for later use. The hESC fragments were then transferred from well 1 to 2 (VS1; Fig. 1) and left for 1 minute. A 20-µl drop of VS2 solution was then aliquoted into the center of a 35-mm Falcon Petri dish. After the 1-minute exposure to VS1 in well two, the hES clumps were transferred quickly to VS2 in well three for 5 seconds (Fig. 1). Using a fresh glass pipette, the clumps were then immediately removed from well three with minimum VS2 and transferred to the fresh 20-µl drop of VS2 in the 35-mm Petri dish for immediate loading into the embryo straw (Fig. 2). This was followed by an air column (~5 mm) and then a column of WS1 (~20 mm; Fig. 2). A pair of cold forceps that had been chilled in ice was then used to hold the straw at the VS2-hESC column firmly while sealing both ends of the straw with a commercial plastic bag heat sealer. After the straw had been completely sealed, it was plunged and stored in either the LLN_2 or VLN_2.

Warming

Warming was performed in a four-well tissue culture dish (Nunclon) with thaw solutions and holding medium, as shown in Figure 3. Straws were removed from VLN_2 or LLN_2 storage and then plunged immediately into a beaker containing water at room temperature. After the columns in the straw had thawed (~5 seconds), the straw was removed from the beaker and swabbed with a 70% isopropanol sterile wipe. Sterile scissors were used to cut off the sealed ends of the straw, and the entire contents of the straw was expelled into WS1 in well one of another four-well dish (Fig. 3) for 1 minute at room temperature. The hESC fragments were then transferred into WS2 in well two (Fig. 3) for 5 minutes at room temperature. Finally, the hESCs were transferred into holding medium in well three (Fig. 3) for 5 minutes. The wash step was repeated in fresh holding medium in well four, and the hES colony fragments were transferred to a new feeder dish plated with mitomycin-C–treated D551/CCL 110 fetal skin fibroblasts.

Evaluation of Post-Thaw hESC Growth, Differentiation, and Colony Scoring

Post-thaw colonies were scored for differentiation by visual inspection. The growth characteristics of hESC colonies in terms of shape, thickness, fragility, and extent of differentia-

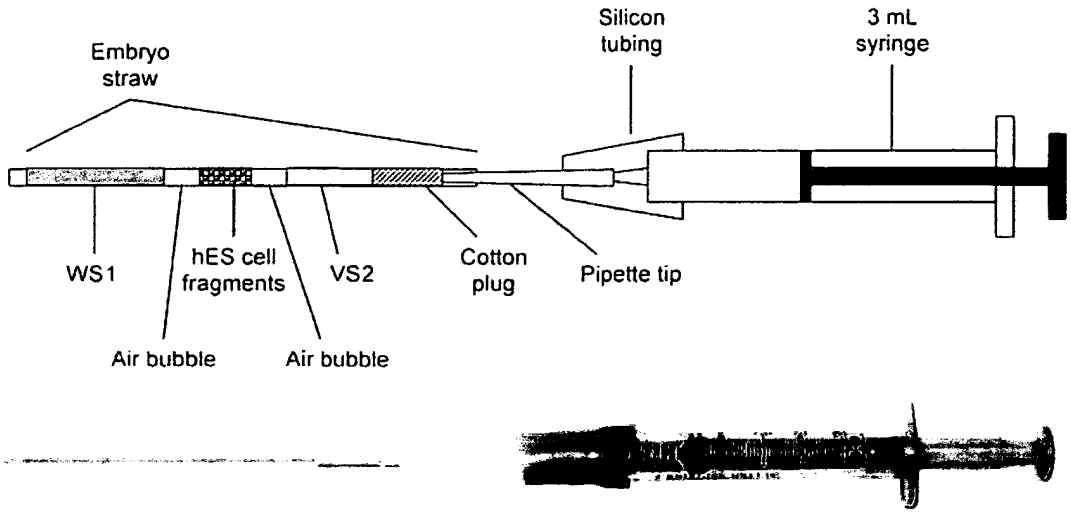

Figure 2. Line drawing and photograph of the loading/unloading syringe and straw device used in the closed-straw vitrification protocol for human embryonic stem cells. Abbreviations: hES, human embryonic stem; VS2, vitrification solution 2; WS1, warm solution 1.

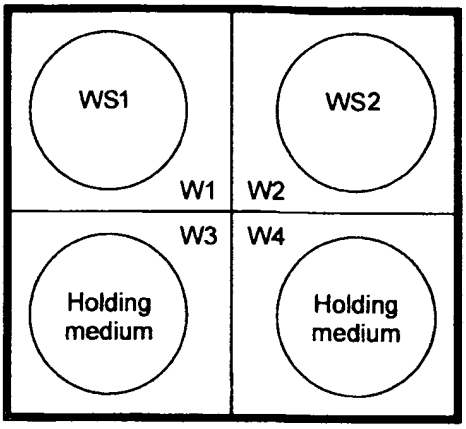

Figure 3. Line drawing of four-well dish showing warming solutions. Abbreviations: WS1, warm solution 1; WS2, warm solution 2; W1, well 1; W2, well 2; W3, well 3; W4, well 4.

tion were carefully recorded at low and high magnifications. Post-thaw colonies were ranked into four classes based on the extent of differentiation. Colonies were assigned as grade A if they remained >80% undifferentiated, grade B if 50%–80% undifferentiated, grade C if <50% undifferentiated, and grade D if either dead or lysed with no growth. Only grade A and B colonies were suitable and used for additional serial passaging.

Statistics

Chi-square analysis was used to determine if differences in the slow freezing and vitrification protocols were significant. The numbers of grade A and B colonies for all hESC lines in each of the seven experimental arms and control arm were pooled for the calculation of P values. The same was done for grade C and D colonies. Pooled grade A and B colonies of one experimental arm were tested against the pooled grade A and B colonies of the control using the chi-square statistic.

Characterization of Post-Thaw/Warmed hESC Colonies

SSEA-4, Tra-1-60, Tra-1-81, and Alkaline Phosphatase Cell-Surface Pluripotency Markers

For immunofluorescence demonstration of stem cell surface markers Tra-1-60 and Tra-1-81, hES colonies were fixed in four-well slide flasks (Becton Dickinson) with 100% ethanol for 20 minutes. For SSEA-4 staining, hES colonies were fixed in 4% paraformaldehyde for 30 minutes. The sources of the monoclonal antibodies for the detection of the markers were as follows: SSEA-4 (MC-813-70), Development Studies Hybridoma Bank (Iowa City, IA); Tra-1-60 and Tra-1-81, gifts from Dr. Peter Andrews, University of Sheffield. Primary antibodies were diluted in PBS (Invitrogen) accordingly, and blocking was performed with 10% normal goat serum (Dako) for 20 minutes. Antibody localization was performed using rabbit anti-mouse immunoglobulin secondary antibody conjugated to flourescein isothiocyanate (Sigma).

Alkaline phosphatase activity was detected with the Vector Red Alkaline Phosphatase Substrate Kit I (Vector Labs, Inc., Burlingame, CA) and viewed with rhodamine excitation and emission filters.

hESCs were routinely tested for markers of pluripotency every 15 passages.

Teratoma Formation in Severely Combined Immunodeficient Mice

Morphologically undifferentiated regions of postwarmed HES-2, HES-3, and HES-4 colonies grown on D551/CCL110 were mechanically dissected into clumps of approximately 300–400 cells each. Approximately 1×10^6 cells were injected with a sterile 27G needle into the thigh muscle of severely combined immunodeficient (SCID) mice. Two SCID mice were injected for teratoma formation for each hESC line. The mice were euthanized 8–12 weeks later, and tumors were dissected and fixed in 4% formaldehyde. Tumors were embedded in paraffin and examined histologically after hematoxylin and eosin staining.

Karyotyping

hES colonies were incubated with 50 mg/ml colcemid solution (Invitrogen) for 2.5 hours at 37°C and in a 5% carbon dioxide in air atmosphere. Cells were trypsinized and washed with PBS (Invitrogen), and pellets were resuspended and incubated with 0.075 M KCl for 30 minutes at 37°C. After treatment with the hypotonic solution, cells were fixed with 3:1 methanol:glacial acetic acid three times and dropped onto precleaned chilled glass slides. Chromosome spreads were Giemsa banded and photographed. At least 20 metaphase spreads and five banded karyotypes were evaluated for chromosomal rearrangements by a qualified cytogeneticist.

Reverse Transcription Polymerase Chain Reaction Analysis

Total RNA was extracted from hESCs with TRIzol reagent (Invitrogen) following the manufacturer's protocol. First-strand synthesis was performed using the SuperScript™ II first-strand synthesis system for reverse transcription polymerase chain reaction (RT-PCR) (Invitrogen). One microgram of first-strand reaction was used for each 50-ml PCR together with 50 pmol of forward and reverse primers. Initial denaturation was carried out at 94°C for 2 minutes and followed by 30 cycles of PCR (94°C for 30 seconds, 55°C for 30

seconds, 72°C for 1 minute) and a final extension cycle at 72°C for 5 minutes. One tenth of each PCR reaction was loaded on a 1.5% agarose gel and size fractionated. The following primers were used: ACTB: product 400 bp 5'-tggcaccacacctttctacaatgagc-3', 5'-gcacagcttctccttaatgtcacgc-3; NANOG: product 493 bp 5'-ggcaaacaacccacttctgc-3', 5'-tgttccaggcctgattgttc-3'; OCT4: product 247 bp 5'-cgrgaagctggagaaggagaagctg-3', 5'-caagggccgcagcttacacatgttc-3'; REX1: product 418 bp 5'-tctagtagtgctcacagtcc-3', 5'-tctttaggtattccaaggact-3'; SOX2: product 370 bp 5'-ccgcatgtacaacatgatgg-3', 5'-cttcttcatgagcgtcttgg-3'.

Real-Time RT-PCR Analysis

Quantitative real-time PCR was performed using TaqMan™ probes from Applied BioSystems Assay (ABI) on Demand™ and Assay by Design™ service. Total RNA was extracted from post-thaw hES colonies using TRIzol reagent and first-strand synthesis performed using the SuperScript™ II first-strand synthesis system for RT-PCR. Markers of pluripotency assayed were *OCT4*, *SOX2*, and *REX1*, and early markers of differentiation assayed were *AFP*, *ND1*, and *BMP4*. Gene expression was normalized to 18S rRNA levels (ABI); equal amounts of input cDNA (25 ng) were used per reaction, and all reactions were performed in triplicate. Real-time PCR analysis was conducted using the ABI PRISM 7000 Sequence Detection System (ABI). These data are summarized in Figure 7 and Table 2. The $2^{-\Delta\Delta Ct}$ method was used to determine normalized target gene expression in post-thaw CS-HSA-VLN_2 and OPS control hES colonies relative to fresh unfrozen hES colonies. Log transformation (base 10) was performed on $2^{-\Delta\Delta Ct}$ values to facilitate graphical representation of relative gene expression data (Fig. 7). Mean C_T values ± standard error of the mean (SEM) values for target genes in fresh colonies, post-thaw CS-HSA-VLN_2, and post-thaw OPS control hES colonies are summarized in Table 2.

Results

The results of post-thaw survival and differentiation for HES-2, HES-3, and HES-4 for the eight different freezing protocols are summarized in Table 1.

Clearly, conventional slow freezing in CVs (CV-FCS-LLN_2 and CV-FCS-VLN_2) was the least effective method for hES cryopreservation and resulted in substantial post-thaw hESC differentiation and cell death (8.2 ± 1.9% to 10.0 ± 10.0% of grade A and B colonies) compared with the experimental and control vitrification arms (75.0 ± 9.7% to 88.3 ± 2.4%, $p < .001$; Table 1). All experimental vitrification protocols gave postwarm survival rates ranging from 75.0 ± 9.7% to 88.3 ± 2.4% (grade A and B colonies), which were as good as controls (79.9 ± 5.0%, grades A and B; Table 1) and unfrozen stock hESC cultures [3].

All vitrified hES clumps that survived the thawing process using the CS approach attached to D551 feeders 24 hours after thaw with little fragmentation (Fig. 4). CS vitrification in LLN_2 or VLN_2 with HSA or FCS proved to be as effective as the OPS-FCS-LLN_2 controls. Thaw-survival differentiation rates (grades A and B) were better when HSA was substituted for FCS in the holding media (80.1 ± 7.7% to 88.3 ± 2.4% versus 75.0 ± 9.7% to 81.7 ± 2.1%). The CS-HSA-VLN_2 ranked first (mean ± SEM of 88.3 ± 2.4% grade A and B colonies from three hESC lines; Table 1), suggesting that this method could replace the existing nonsterile OPS method. Postwarmed vitrified colonies for experimental and control arms had to be passaged a bit earlier at day 6 or 7 before differentiation, unlike unfrozen colonies (days 7 and 8).

Post-thawed hESC colonies tested positive for typical hESC-surface markers, such as Tra-1-60, Tra-1-81, and SSEA-4 (Fig. 5), and strong alkaline phosphatase activity was also detected (Fig. 5). Teratomas comprising all three primordial germ layers were formed in SCID mice after intramuscular thigh injection of undifferentiated post-thawed hESC colony fragments, confirming pluripotency (Fig. 6). Post-

Table 2. Mean C_T ± SEM $2^{-\Delta\Delta CT}$ values for post-thaw CS-HSA-VLN_2 colonies, OPS control colonies, and fresh unfrozen hES colonies

	Mean C_T ± SEM		Mean $2^{-\Delta\Delta CT}$		
Target gene	Unfrozen fresh hESCs	CS-HSA-VLN_2	OPS Controls	CS-HSA-VLN_2	OPS Controls
OCT4	21.073 ± 0.001	21.732 ± 0.248	20.825 ± 0.027	0.321	0.328
SOX2	21.798 ± 0.104	22.442 ± 0.146	22.079 ± 0.053	0.364	0.246
REX1	23.355 ± 0.000	23.940 ± 0.038	23.343 ± 0.072	0.312	0.353
AFP	39.872 ± 0.032	39.402 ± 0.013	29.412 ± 0.020	1.027	686.921
ND	37.183 ± 1.183	32.674 ± 0.319	31.846 ± 0.012	13.576	12.059
BMP4	26.072 ± 0.071	26.769 ± 0.066	25.583 ± 0.063	0.807	0.544

Abbreviations: CS, closed straw; hES, human embryonic stem; HSA, human serum albumin; OPS, open pulled straw; SEM, standard error of the mean; VLN_2, vapor phase of liquid nitrogen.

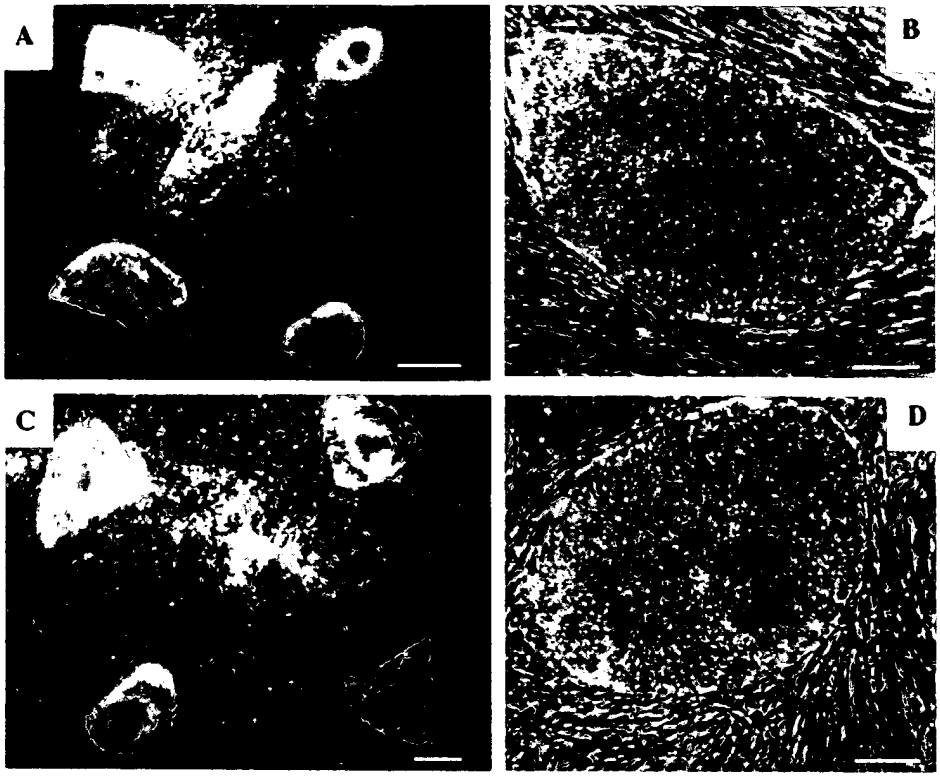

Figure 4. (A): Bright field light micrograph (low magnification) of undifferentiated 7-day-old postwarm control HES3 (23P) colonies (OPS-FCS-LLN_2) on a human feeder cell layer (D551). **(B):** Phase-contrast light micrograph (high magnification) of an undifferentiated 5-day-old postwarm HES3 (23P) colony (OPS-FCS-LLN_2) on a human feeder cell layer (D551). **(C):** Bright-field light micrograph (low magnification) of undifferentiated 7-day-old postwarm HES3 (23P) colonies (CS-HSA-VLN_2) on a human feeder cell layer (D551). **(D):** Phase-contrast light micrograph (high magnification) of an undifferentiated 5-day-old postwarm HES3 (23P) colony (CS-HSA-VLN_2) on a human feeder cell layer (D551). Scale bars in **(A, C)** = 1 mm; in **(B, D)** = 100 μm. Abbreviations: CS, closed straw; FCS, fetal calf serum; HSA, human serum albumin; LLN_2, liquid phase of liquid nitrogen; OPS, open pulled straw; VLN, vapor phase of liquid nitrogen.

thawed hESC colonies also displayed normal banded karyotypes (Fig. 6). Chromosomal breakages and other abnormalities were not detected at the first passage after warming as well as subsequent passages, and hESC colonies continued to express other hES-specific marker genes, such as *POU5F1*, *SOX2*, *NANOG*, and *REX1* (Fig. 5).

Real-time PCR analysis conducted on day 6 CS-HSA-VLN_2, day 6 post-thaw OPS control colonies, and fresh unfrozen colonies indicated that *OCT4*, *SOX2*, and *REX1* expression levels in CS-HSA-VLN_2 post-thaw colonies were comparable with those of post-thaw OPS controls (Table 2). However, a slight decrease in *OCT4*, *SOX2*, and *REX1* expression in post-thaw CS-HSA-VLN_2 and OPS control colonies was evident when expression levels of these markers of pluripotency were compared with the expression levels of these genes in fresh, unfrozen hES colonies (Fig. 7). Post-thaw CS-HSA-VLN_2 colonies also showed an increase in *ND1* expression, indicating neural differentiation, whereas post-thaw OPS control colonies showed large increases in both *AFP* and *ND1* (Fig. 7, Table 2). This suggested that colony differentiation was more extensive in post-thaw OPS control colonies than in post-thaw CS-HSA-VLN_2 cultures. We could not harvest enough post-thaw colonies frozen by the CV-FCS-LLN_2 and CV-FCS-VLN_2 methods for real-time PCR analysis because of extensive cell death. Overall, quantitative real-time PCR results matched well with our morphological scoring scheme for colony differentiation.

Discussion

Monolayer cell cultures grown on plastic and subsequently trypsinized into single cells for serial passaging are traditionally cryopreserved using the conventional slow controlled-rate freezing approach in sealed CVs with 90%:10% FCS:DMSO and stored in the LLN_2. This method was found to be unsuitable for hESCs in both this study and that of Reubinoff et al. [11]. hESCs are colony-forming social cells that do not survive well and undergo differentiation sponta-

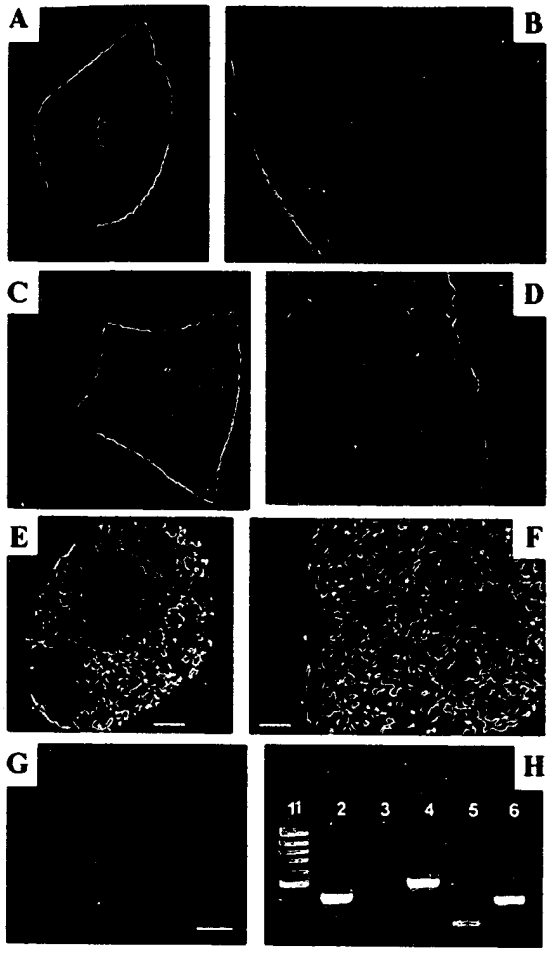

Figure 5. hESC marker characterization of postwarm (closed straw–human serum albumin–vapor phase of liquid nitrogen) (25P) HES3 colonies on human (D551) feeder layers. Tra-1-60 cell-surface marker staining of whole human embryonic stem (hES) colony. Scale bar = 500 μm **(A)**. High magnification of Tra-1-60 surface marker staining. Scale bar = 100 μm **(B)**. Tra-1-81 cell-surface marker staining of whole hES colony. Scale bar = 2,000 μm **(C)**. High magnification of Tra-1-81 surface marker staining. Scale bar = 100 μm **(D)**. SSEA-4 cell-surface marker staining of whole hES colony. Scale bar = 500 μm **(E)**. High magnification of SSEA-4 surface marker staining. Scale bar = 100 μm **(F)**. Alkaline phosphatase activity of whole hES colony. Scale bar = 400 μm **(G)**. Reverse transcriptase polymerase chain reaction pluripotency marker analysis. Lane 1, Fermentas 100-bp ladder; lane 2, *ACTB*; lane 3, *REX1*; lane 4, *NANOG*; lane 5, *OCT4*; lane 6, *SOX2* (H).

neously when dispersed into single cells and as such need to be propagated in small clumps to retain an undifferentiated phenotype. This characteristic of hESCs could explain low recovery and elevated differentiation rates observed when hESCs were cryopreserved with controlled-rate freezing protocols. It may be difficult to optimally induce ice crystal formation in the clumps of hESCs in the prolonged slow stepwise controlled-rate freezing protocol because of the adherent tight nature of the cells. Transmission electron microscopy ultrastructural studies have shown many tight junctions and gap junctions in human ES cell colonies [12]. Also, several gap junction proteins and cell adhesion molecules (for example, connexin 43 and claudin 6) are very highly expressed in human ES cells [13]. Furthermore, low cloning efficiencies reported for human ES cells indicate that human ES cells do not survive well as single cells [14]. These data highlight the importance of cell–cell adhesion in human ES cell colonies. Ice crystal formation outside of the cells during controlled-rate cooling may disrupt cell–cell adhesion and thus explain the low recovery and increased differentiation rates when controlled-rate freezing methods are used to cryopreserve hESCs.

The results of this study indicate that hESCs may be successfully cryopreserved using the CS vitrification approach. In vitrification, it is important to induce glass formation by bringing the cells into rapid contact with the LN_2 once they are in the vitrification solutions. This is necessary because of the high concentration of cryoprotectants used, which can be toxic to the cells. Because heat-sealing of the straws delays this process, two steps were incorporated to reduce this toxicity in the CS method of this study. The column containing hESC fragments was held by a pair of prechilled (chilled in ice) broad forceps and sandwiched by VS2 and WS1 columns so as to protect the hESC fragments during heat sealing. Also, the prechilled forceps allow rapid permeation of the cold temperature through the wall of the straw.

Additionally, the WS1 column helps to flush out any hESC fragments remaining in the straw during the warming process. It must be noted that for this entire study, there was no loss of hESC fragments in the CS method after warming. Thus, for the CS method to work well, the correct brand of straw with the right wall thickness used, as well as the speed of sealing, may be important. The total time taken for transfer of the hESC fragments to VS2 and the sealing of the straws and the plunging into LN_2 should not exceed 120 seconds. In this study, the average duration was 100 seconds. It is important that the straws be completely sealed to ensure sterility as well as prevent the danger of explosion due to the seepage of LN_2.

The vitrification technique is quick and rapid, and unlike slow gradient freezing, it minimizes the exposure time of the cells to low temperatures outside the normal physiological temperature range. Vitrification also circumvents problems associated with ice formation, cell dehydration, and the control of freezing rates. Furthermore, vitrification totally eliminates the formation of ice crystals, unlike slow gradient freezing, where substantial ice nucleation and

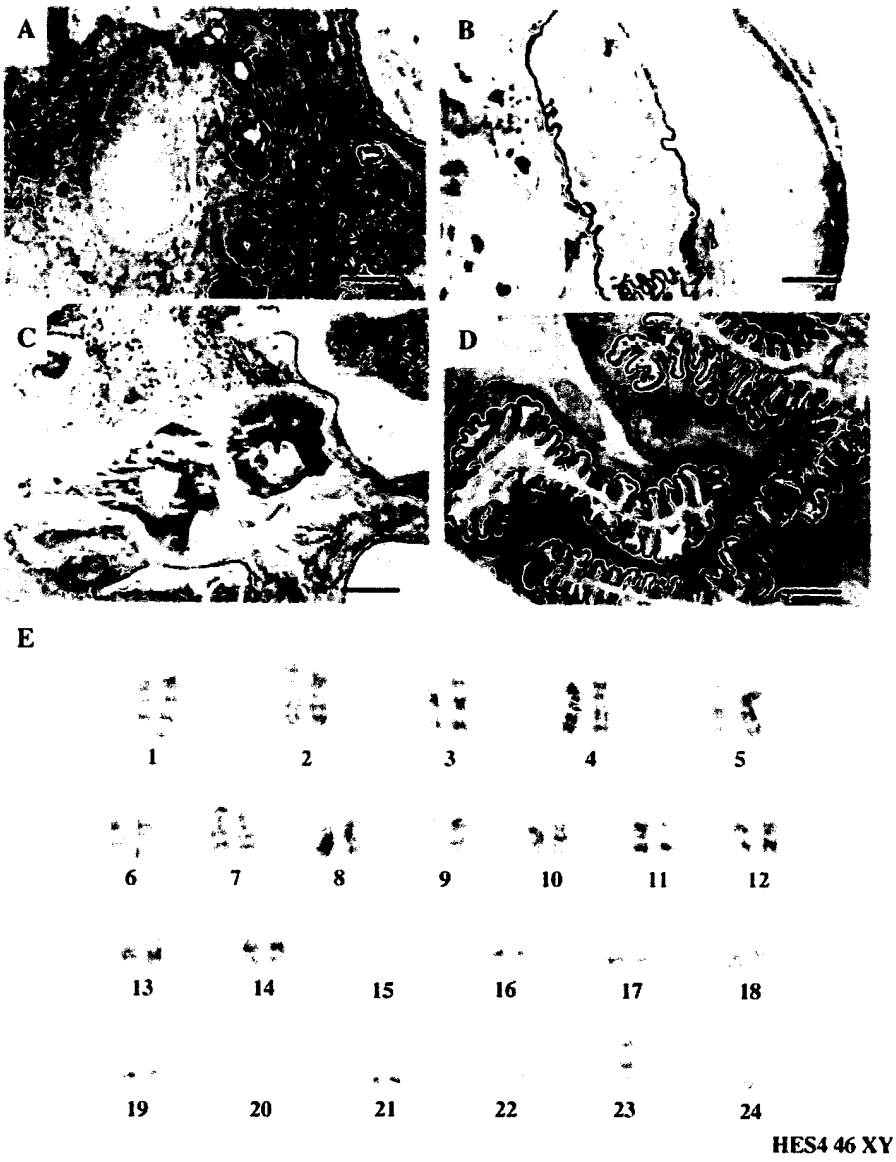

Figure 6. Tests for pluripotency and karyotypic stability of postwarm (CS-HSA-VLN_2) (25P) HES4 colonies on human (D551) feeder layers. Teratoma sections showing primitive cartilage and neural rosettes (**A**), cartilage and cystic epithelium (**B**), bone (**C**), and developing gut (**D**). All scale bars = 200 μm. Normal 46 XY metaphase for postwarm (CS-HSA-VLN_2) (25P) HES4 colonies on human (D551) feeder layers (**E**). Abbreviations: CS, closed straw; HSA, human serum albumin; VLN_2, vapor phase of liquid nitrogen.

crystallization can occur outside the cells. Also, to minimize cryoprotectant toxicity, all procedures were carried out at room temperature.

The CS method is a safe and effective approach for hESC cryopreservation; the results are highly reproducible, as reflected by the consistency of the replicates, unlike some other vitrification protocols [15, 16]; and the method is cheap, not requiring expensive programmable freezing machines. CS-vitrified hESC colonies continued to express markers of pluripotency, formed teratomas in SCID mice, and maintained normal diploid karyotypes after warming and subsequent culture, confirming that they maintain their bona-fide stem cell properties after vitrification warming. Post-thaw 6-day-old CS-vitrified hESCs did not show elevated AFP gene expression compared with unfrozen colonies, suggesting that CS vitrification and thawing procedure did not induce endodermal differentiation in hESCs. *OCT4*, *SOX2*, and *REX1* expression levels in 6-day-old CS-vitrified hES colonies were similar to those of OPS controls but only slightly reduced compared with fresh unfrozen hES

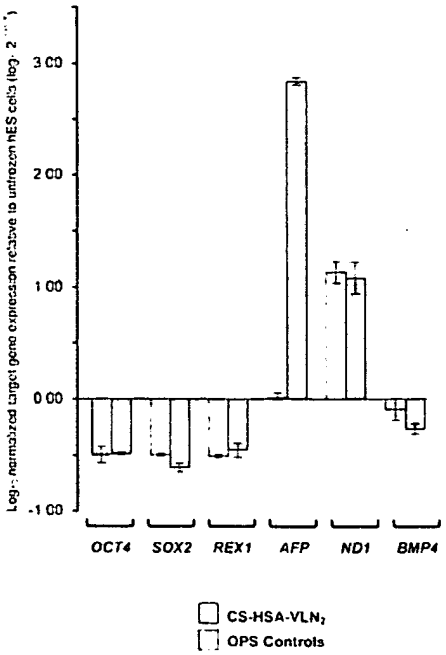

Figure 7. Comparative assessment of markers of pluripotency and differentiation in post-thaw CS-HSA-VLN_2 colonies and open pulled straw control colonies with real-time PCR. Normalized *OCT4*, *SOX2*, *REX1*, *AFP*, *ND1*, and *BMP4* expression relative to fresh, unfrozen hES colonies. Abbreviation: hES, human embryonic stem.

colonies. Zona-free hatched murine blastocysts have also been successfully vitrified in CSs sealed at both ends [17], and, more recently, rabbit zona-intact blastocysts were effectively vitrified in standard plastic straws sealed at both ends as a model for human blastocysts [18].

The CS vitrification method that we describe gives excellent recovery of hESC colonies after thawing with low differentiation rates, comparable with the results of Reubinoff et al. [11]. Vitrified-warmed colonies were observed to differentiate slightly earlier than unfrozen control colonies. These findings are consistent with those reported by Reubinoff et al. [11].

FCS is a major constituent of the cryoprotectants conventionally used in the OPS method [11] and other hESC-freezing protocols. It is also undesirable in a CGMP-compliant hESC culture protocol. This study showed that a human-based protein (HSA) can successfully be substituted for this mixture of xeno-proteins. The CS method in this study therefore describes a totally xeno-free approach for hESC cryopreservation.

The results also demonstrated that CS vitrification can be performed in either the LLN_2 or VLN_2. It has been well documented that LN_2 not only serves as a refrigerant but can also act as a vehicle for the transmission of viruses, bacteria, fungi, and animal cells. Reports that infectious viruses were found in LN_2 and thus should be treated as a biohazard [19] are indicative of the potential dangers of the liquid phase of LN_2. As models for human and animal viral pathogens, three bovine viruses, bovine viral diarrhea virus (BVDV), bovine herpes virus (BHV), and bovine immunodeficiency virus (BIV), were used to study the potential for their transmission by experimentally contaminated LN_2 to embryos frozen and stored in open freezing CVs [20]. Bovine embryos in a mixture of 20% ethylene glycol, 20% ME_2SO, and 0.6% sucrose were vitrified in either unsealed standard 0.25-ml or modified pulled straws or in open plastic CVs and then plunged into the contaminated LLN_2. Postwarmed testing of a pool of 83 batches of embryos showed that 13 of 61 (21.3%) exposed to BVDV and BHV tested positive for BVDV and BHV, whereas 22 batches exposed to BIV tested negative for BIV. All control embryos vitrified in sealed CVs and straws were free from viral contamination [20]. The submersion of OPS straws and screw-capped plastic CVs in the liquid phase allows for contact between contaminated LN_2 and the sample. Condensation of the atmosphere within the tube creates a vacuum that can draw in the LN_2, and any contaminants in the LN_2 may thus contaminate the sample. OPS vitrification [11] involves direct contact of the hESC clumps with LN_2, thus substantially elevating the risk of contamination with adventitious agents in LN_2. However, CS vitrification in LN_2 reduces the possibility of hESC contamination, because the straws are sealed at both ends, whereas vitrification of hESCs in the vapor phase above the levels of LN_2 eliminates totally the possibility of the contamination of cells with adventitious agents and the cross-contamination with other cell types in the same storage vessel. This method also accommodates the temporary storage and shipping of hESCs in LN_2 dry shippers. We have observed that the temperature of LN_2 dry shippers is constantly below −132°C, the theoretical glass transition temperature of water; cells stored below this temperature are estimated to survive for hundreds of years [7]. Therefore, the CS vitrification protocol with storage in LN_2 vapor we describe is an improvement over previous hESCs vitrification protocols.

We note, however, that our method of passaging hESCs involved manual manipulation of individual colonies. A major drawback of this method is the difficulty in generating sufficient numbers of cells for clinical application. In addition, smaller volumes and fewer numbers of cells are cryopreserved using the vitrification technique in straws. However, we feel that, in combination, these techniques will be particularly useful for the cryopreservation of critical very-early-passage hES stock and for hESC lines that are not amenable to bulk culture protocols.

The CS vitrification protocol described in this study for hESC cryopreservation may also work for future hES-directed tissues, such as clumps of islet-like cells, and as such will be very useful in complementing efforts in starting hESC banks and the creation of new xeno-free hESC lines for therapeutic application.

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Erratum

AN EFFICIENT AND SAFE XENO-FREE CRYOPRESERVATION METHOD FOR THE STORAGE OF HUMAN EMBRYONIC STEM CELLS

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On page 784 and 788 of the September 2004 issue, "An Efficient and Safe Xeno-Free Cryopreservation Method for the Storage of Human Embryonic Stem Cells," by Mark Richards, Chui-Yee Fong, Shawna Tan, Woon-Khiong Chan, and Ariff Bongso, Table 2 and Figure 7 were incorrectly displayed. Here we reprint the table and the figure in their entirety. The online version has been corrected.

Table 2. Mean $C_T \pm$ SEM $2^{-\Delta\Delta CT}$ values for post-thaw CS-HSA-VLN_2 colonies, OPS control colonies, and fresh unfrozen hES colonies

	Mean $C_T \pm$ SEM			Mean $2^{-\Delta\Delta CT}$	
Target gene	**Unfrozen fresh hESCs**	**CS-HSA-VLN_2**	**OPS Controls**	**CS-HSA-VLN_2**	**OPS Controls**
OCT4	21.073 ± 0.001	21.732 ± 0.248	20.825 ± 0.027	0.321	0.328
SOX2	21.798 ± 0.104	22.442 ± 0.146	22.079 ± 0.053	0.364	0.246
REX1	23.355 ± 0.000	23.940 ± 0.038	23.343 ± 0.072	0.312	0.353
AFP	39.872 ± 0.032	39.402 ± 0.013	29.412 ± 0.020	1.027	686.921
ND	37.183 ± 1.183	32.674 ± 0.319	31.846 ± 0.012	13.576	12.059
BMP4	26.072 ± 0.071	26.769 ± 0.066	25.583 ± 0.063	0.807	0.544

Abbreviations: CS, closed straw; hES, human embryonic stem; HSA, human serum albumin; OPS, open pulled straw; SEM, standard error of the mean; VLN_2, vapor phase of liquid nitrogen.

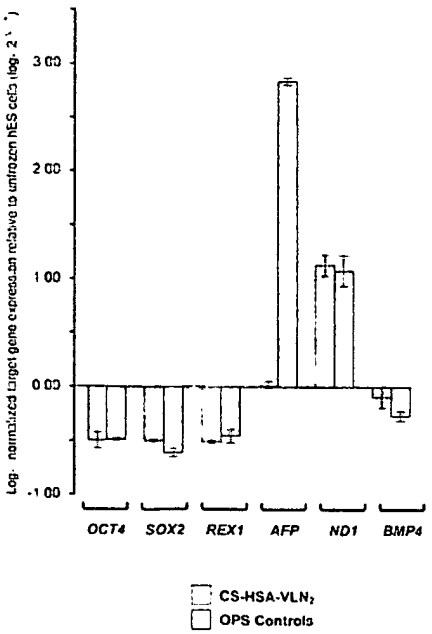

Figure 7. Comparative assessment of markers of pluripotency and differentiation in post-thaw CS-HSA-VLN_2 colonies and open pulled straw control colonies with real-time PCR. Normalized *OCT4*, *SOX2*, *REX1*, *AFP*, *ND1*, and *BMP4* expression relative to fresh, unfrozen hES colonies. Abbreviation: hES, human embryonic stem.

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